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AWARD NUMBER: W81XWH-04-1-0438

TITLE: Met Nuclear Localization and Signaling in Breast Cancer

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REPORT DATE: May 2006

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
May 2006	Annual Summary	2 Apr 05 – 1 Apr 06

4. TITLE AND SUBTITLE

Met Nuclear Localization and Signaling in Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0438

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Yale University
New Haven, Connecticut 06520-8047

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Some breast cancer cases in our previous immunohistochemical studies show Met expression in the nucleus. Given nuclear localization of other receptor tyrosine kinases, we proceeded to investigate Met. Nuclear Met is seen in numerous cell lines and in germinal regions of many tissues using 4 unique antibodies. Cell fractionation reveals a 60kDa band recognized by C-terminal Met antibodies that is present independent of HGF treatment. GFP fusion proteins of the cytoplasmic domain of Met transfected into HEK293 cells are found in the nucleus while the full length Met-GFP fusion is membranous. Further deletions of the Met-GFP fusions identify a region of the juxtamembrane domain required for nuclear translocation. In a CaCo2 cell line model for epithelial maturation, we find that Met is initially nuclear, and then becomes membranous, after confluence. Nuclear translocation can then be induced by wounding the cell monolayer. This work suggests processing of the Met receptor, analogous to ErbB4, resulting in the release of the cytoplasmic domain and its translocation to the nucleus during stages of cell cell growth and proliferation.

15. SUBJECT TERMS
c-Met, receptor tyrosine kinase, cleavage

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
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Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Introduction:
Met (the c-met gene product) is a receptor tyrosine kinase for the HGF ligand that is expressed on the surface of epithelial and endothelial cells. The primary 150kDa transcript is partially glycosylated to form the 170kDa Met precursor that is then cleaved forming a heterodimer consisting of two subunits (45kDa and 150kDa) joined by disulfide bonds. The smaller alpha subunit is entirely extracellular, whereas the larger beta subunit traverses the plasma membrane and includes a juxtamembrane region responsible for negatively regulating Met, a tyrosine kinase domain with intrinsic kinase activity, and a C-terminal region that provides binding sites for target substrates (for review see (1)). Activation of the docking site triggers signaling cascades such as Gab1, Grb2 and PI3K, leading to proliferation, scattering, increased motility, invasion and branching morphogenesis (reviewed in (2)). Met has also been shown to be activated in the absence of HGF ligand. Constitutive activation of Met can occur via mutations in the cytoplasmic domain and are associated with the genesis and progression of some human tumors (3).
A growing list of membrane proteins have been found to translocate to the nucleus. Members of the epidermal growth factor (EGF) and fibroblast growth factor (FGF) family of receptor tyrosine kinases have been shown to translocate to the nucleus as either intact receptors or in the case of ErbB4, as a truncated fragment, activating transcription of target genes (4) (5). Other membrane proteins that undergo similar cleavage events and subsequent nuclear localization include Notch, APP, CSF-1, E-cadherin and CD44 (6) (7) .
A previous immunohistochemical study in our lab on over 600 cases of breast cancer showed that expression of the Met cytoplasmic domain, and not the extracellular domain was correlated with poor patient outcome in lymph node negative breast carcinomas (8). This difference is not easily explained, but one possibility is a processing event where a C-terminal fragment is present in the absence of the N-terminus.

Body:
The goal of this study is to verify the presence of the Met receptor in the nucleus of cell lines. Following confirmation of this phenomenon, the goal becomes to determine the mechanism by which Met translocates to the nucleus and the function this plays in HGF-Met signaling.

The original statement of work was as follows:

Task 1. To confirm nuclear localization of the cytoplasmic domain of the Met receptor in HMEC cells, Months 0-24:
- a. Construction and transfection of GFP-tagged Met cytoplasmic domain, Months 0-3
- b. Subcellular fractionation, Months 3-6
- c. Identification of Met antibodies for use on tissue microarray of normal and cancerous cells, Months 12-24

Task 2. Definition of the domain responsible for translocation to the nucleus, Months 6-30):
- a. Construct and transfection of truncated versions of GFP-tagged cytoplasmic Met, Months 6-12
- b. Subcellular fractionation, Months 12-18

c. Characterization of turnover rate, 18-30

Task 3. Definition of the cleavage domain and cleavage site, Months 18-36:

a. Tag full length Met and transfect into cells to verify cleavage, Months 18-21
b. Bioinformatics to determine and test protease candidates, Months 21-27
c. Create mutant Met unable to be cleaved, Months 27-30
d. Characterize mutant and wild type effect on cells using growth assays, Months 30–36+

The purpose of Task 1 was to confirm the nuclear localization of Met. Immunohistochemistry, using an anti-peptide polyclonal antibody to the C-terminus of the Met receptor, shows Met in the cytoplasm and nucleus of normal and cancerous tissues rather than the previously reported predominantly membrane staining (Figure 1a)(9,10). Normal colon, skin and testis show similar nuclear staining of Met in germinal tissue layers and aberrant expression throughout cancerous tissue. The transition to Met over-expressing cancers is most dramatically seen in lymphoma tissue where Met is completely absent in the normal lymph node tissue and localized almost exclusively to the nucleus in cancerous tissue.

To confirm that the nuclear localization of Met expression was not an artifact seen only in formalin fixed, paraffin embedded tissue, a series of cell lines were stained with four different antibodies to the cytoplasmic domain. The antibodies selected were polyclonal antibodies C12, C28, and CVD13 to peptides very near or including the C-terminus of Met, and 3D4, a monoclonal antibody to the tyrosine kinase domain. Mammary epithelial lines HMEC and MCF10-A, melanoma cell lines Mel1241 and Mel1335 (data not shown), and epidermoid carcinoma line A431 (Figure 1b) all showed nuclear expression of Met to varying degrees depending on the antibody used. HEK293 and NIH3T3 cell lines express low levels of Met yet also showed nuclear staining equivalent to those known to express higher levels of Met. DO24, a monoclonal antibody to the extracellular domain of Met, shows membranous staining of HMEC, MCF-10A, HEK293 and A431 cell lines only in dense regions of the cell colony.

Since nuclear staining of Met was seen in cell lines previously considered low expressers or negative for Met, a northern blot was done to confirm the presence of Met in these cell lines. Using a radioactively-labeled probe representing 450 base pairs at the 3' end of the translated sequence of Met, a 9.5kb transcript was detected in all cell lines. The smaller 7.5kb variant of Met was also seen in all cell lines except NIH3T3 and Mel1335 (Figure 2). No smaller isoforms were seen. Thus, although some lines (NIH 3T3) have previously been used as negative controls for Met transfection (11), full length message is observed for each cell line. Furthermore, this also suggests that the protein is not derived from a novel alternative splicing variant.

To determine if Met is present in the nucleus, cell fractionation was done to separate the nuclear fraction from the membranous and cytoplasmic fractions using standard sucrose gradient-based methods. The epidermoid carcinoma cell line A431 was used as a model. Separation of the cytoplasm from the nucleus resulted in a 60kDa protein that is reactive with Met antibody localizing to the nuclear fraction, while the 145kDa Met receptor remained in the cytoplasmic fraction in both A431 (high Met expresser) and HEK293 (low Met expresser) cell lines (Figure 3a). Tubulin is shown as a control for the cytoplasmic fraction and lamin serves as a control for

the nuclear fraction. Since the 60 kD fragment has not previously been reported, we used a series of two polyclonal antibodies and one monoclonal antibody to prove the band is Met and not a cross reacting protein. Western blotting of A431 lysates from this line showed the expected band at 145 kDa and no band at 60kDa. Immunoflourescence detected low levels of nuclear Met in A431 cells suggesting that the nuclear fragment may need to be enriched in some manner to detect it on a western blot, this is in contrary to subcellular fractionation results as fractionation served to enrich the amount of nuclear protein seen on a western blot. When A431 cells were serum starved and treated with ALLN, a proteasome inhibitor, a band that co-migrates with 60kDa markers is detected by three different antibodies to Met (Figure 3b). To further prove specificity we immunoprecipitated Met from total cell lysates of ALLN treated and untreated A431 cells as well as untreated HEK293 cells and NIH3T3 cells. All cell lines show a the cleaved Met precursor (190kDa), full length Met (145kDa) and a band of 60kDa (Figure 3c) when immunoprecipitated with either a monoclonal or a polyclonal Met antibody. As seen in subcellular fractionation, the presence of the 60kDa fragment in untreated cells is again due to the enrichment capability of the assay. Immunoprecipitation of NIH3T3 cells, which do not express Met at the membrane, does not pull down full length Met when immunoprecipitated with the C12 antibody, however the 3D4 antibody pulls down the uncleaved Met precursor. Additionally, the 3D4 antibody shows a band slightly higher than the 60kDa fragment that also appeared faintly in the no antibody control lane. Together, this data supports the presence of a 60kDa fragment of Met in the nucleus.

Task 2 was to define the domain responsible for nuclear translocation. To verify that a region of the cytoplasmic domain is present in the nucleus, Met and N-terminally truncated forms of the cytoplasmic domain of Met were cloned into a GFP fusion vector so that GFP was located on the C-terminus of the protein. A full length Met protein fusion, a fusion with the cytoplasmic domain truncated at the transmembrane/intracellular junction (K956), three constructs truncated after tyrosine residues in the juxtamembrane region (D972, R1004, P1027), the tyrosine kinase domain (I1084) and a truncated tyrosine kinase domain (L1157) were constructed. Each Met construct had a predicted molecular weight of 49, 47, 43, 41, 35 and 27 kDa, respectively, the GFP tag is an additional 30kDa. All of the constructs shown in a schematic in Figure 5a, were then transiently transfected into HEK293 cells. A Western blot of HEK293 cells transiently transfected with the cytoplasmic domain of Met confirmed the presence of the GFP-tagged Met construct (Figure 5b). The full length Met-GFP fusion localized to the plasma membrane of HEK293 cells as expected (Figure 5c). Surprisingly, this construct has not been seen in the nucleus under any conditions, including treatment with HGF. HGF is the ligand for Met, and its interaction with the extracellular domain of Met triggers dimerization and phosphorylation of the receptor and activation of a number of downstream signaling pathways including the MAPK pathway (17). In order to assess the effect of HGF on the 60kDa fragment we treated two model cell lines with HGF over a 24 hour time course. Treatment with HGF did not induce the appearance of the 60kDa fragment. Figure 4 shows that although HGF is activating downsteam signaling, as shown by phosphorylation of ERK1/2, the 60kDa fragment is not induced. However, treatment with ALLN, which inhibits proteolysis, substantially increases the amount of the 60kDa fragment in both cell lines in both the presence and absence of HGF (Figure 4). This may be due to the construction of an unnatural state of forced overexpression as nuclear expression of Met is not readily seen in HT-29 cells which overexpress Met (data not shown). As we have yet to determine the mechanism by which Met enters the nucleus another possibility

for the lack of nuclear Met-GFP is that the transfected cells were not properly stimulated to express nuclear Met-GFP. However, constructs of the cytoplasmic domain of Met localized to the nucleus of cells. Loss of the juxtamembrane region caused Met-GFP tyrosine kinase domain constructs to be partially excluded from the nucleus of cells and loss of the N-terminus of the tyrosine kinase domain led to complete exclusion from the nucleus. (Figure 5c). The same localization results were observed in transfections of MCF-10A cells (data not shown). Transient transfections of Met-GFP constructs confirm the ability of the cytoplasmic domain of Met to enter the nucleus. The region of the juxtamembrane domain preceding the tyrosine kinase domain (P1027-I1084) appears to be necessary for nuclear localization of the cytoplasmic domain. As we have yet to identify the conditions required for stimulating nuclear translocation of the Met-GFP construct, we have not been able to investigate turnover rate of the fragment.

The goal of Task three was to further describe the cleavage domain and site of Met. Although there has been no progress on identifying a stimulant for Met cleavage, it has been observed that this phenomenon is cell density dependent. CaCo2 are a model line for cells maturation and differentiation as a function of time and density when grown in culture (12,13). Preconfluent CaCo2 cells show nuclear and cytoplasmic expression of Met as seen in Figure 6a. CaCo2 cells matured for three days past confluence show a more distinct membranous as well as cytoplasmic expression of Met (Figure 6b). Similar relocalization patterns were obtained with A431 cells (data not shown), however, it required A431 cells to be grown for a minimum of ten days past confluence to detect regions of membranous Met expression. This suggests that nuclear expression of Met may be detected only in cells in a state of rapid proliferation or in a less differentiated state, a hypothesis consistent with the expression patterns of Met in proliferative areas of normal tissues in Figure 1a and in moderately or poorly differentiated cancers. In accordance with this hypothesis, a wound healing assay on these highly confluent cells causes Met to translocate from the membrane to the nucleus at the wound edge (Figure 7). Bioinformatics suggest a possible gamma-secretase cleavage site located in the transmembrane region of Met, however, attempts at inhibiting this phenomenon with gamma-secretase and caspase inhibitors have been unsuccessful thus far. Attempts at making an uncleavable Met have not been made at this time as we have not yet identified the proper conditions for cleavage of the Met-GFP construct.

Key Research Accomplishment:

- Verification of Met in the Nucleus
- Identification of region responsible for Met nuclear localization
- Identification of environmental stimuli for Met to translocate to the nucleus

Reportable Outcomes:

Poster Presentation: San Antonio Breast Cancer Research Symposium, December 2004
Poster Presentation: Era of Hope, June 2005
Poster Presentation: FASEB, August 2005
Poster Presentation: AACR, April 2006
Publication in press: *Met, the HGF Receptor, Localizes to the Nucleus in Early Stages of Maturation,* Cancer Research

Conclusions:

Here, I demonstrate for the first time that a C-terminal fragment of the HGF receptor Met localizes to the nucleus in a ligand-independent manner. Using a series of monoclonal and polyclonal antibodies to the C-terminus of Met, I have shown that Met expression is present not only at the membrane, but also in the cytoplasm and nucleus in seven cell lines and predominantly in germinal regions in a range of tissues from normal organs. Nuclear translocation is reconstituted by transfection of GFP fusions with Met that migrate or do not migrate to the nucleus, dependent on the composition of the fusion. Cell lines expressing nuclear Met have either a mesenchymal phenotype or are on the leading edge of epithelial clusters or coincide with a less differentiated state. The maturation and wounding of the Caco2 cells provides the best model to illustrate this phenomenon.

All cell lines expressing Met in the nucleus show Met transcripts by Northern Blot. Western blotting reveals a 60kDa band recognized by antibodies to the C-terminus of Met that localizes to the nucleus. The appearance of a smaller protein recognized by Met antibodies in the nucleus and the lack of a smaller transcript by Northern Blot suggests that this fragment is derived from the full length Met receptor by a processing event. The increase in amounts of the fragment in the presence of ALLN provides further evidence for a processing event. Although no nuclear localization sequence has been identified, serial deletion constructs of Met isolate a region of the juxtamembrane domain (P1027-I1084) that is required for nuclear localization of the cytoplasmic fragment of Met. This fragment is not an alternative splice as shown by Northern blotting and is most likely the product of a cleavage event as the full length Met-GFP fusion does not localize to the nucleus.

The expression of nuclear Met in germinal tissue layers and at the periphery of some epithelial clusters suggests that nuclear Met may be associated with a mesenchymal or germinal phenotype. The reduction of nuclear expression of Met in maturing CaCo2 cells and the translocation of Met to the nucleus upon wounding suggests that nuclear Met might also be a trait of less differentiated cells. The presence of Met in the nucleus indicates that it may play a role in enhancing signaling of the full length Met receptor or may be indicative of a novel signaling pathway. Regardless, further studies on the function of Met in the nucleus will be important to fully understanding the Met signaling pathway. This is particularly important in light of the fact that a number of pharmaceutical companies are in various phases of testing Met targeted therapies.

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Appendix 1: Figures

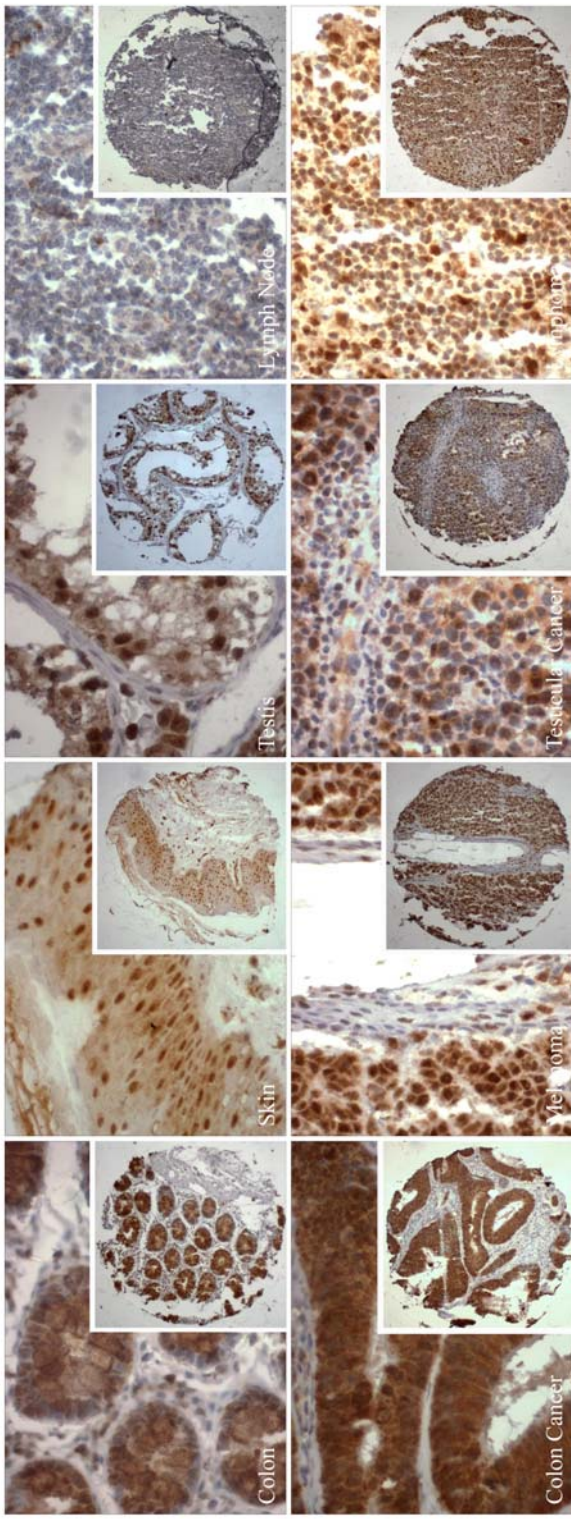

Figure 1a

Figure 1b

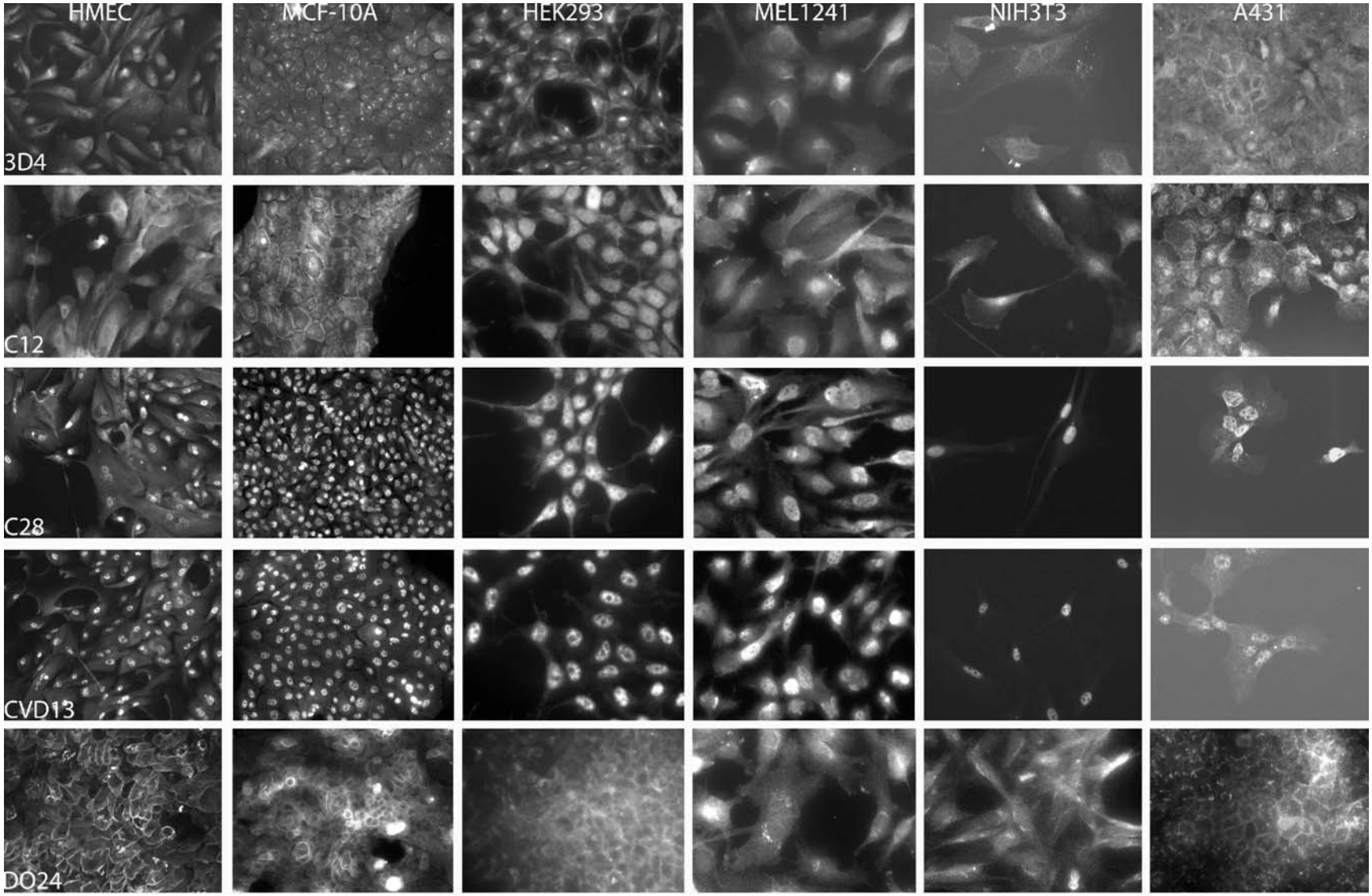
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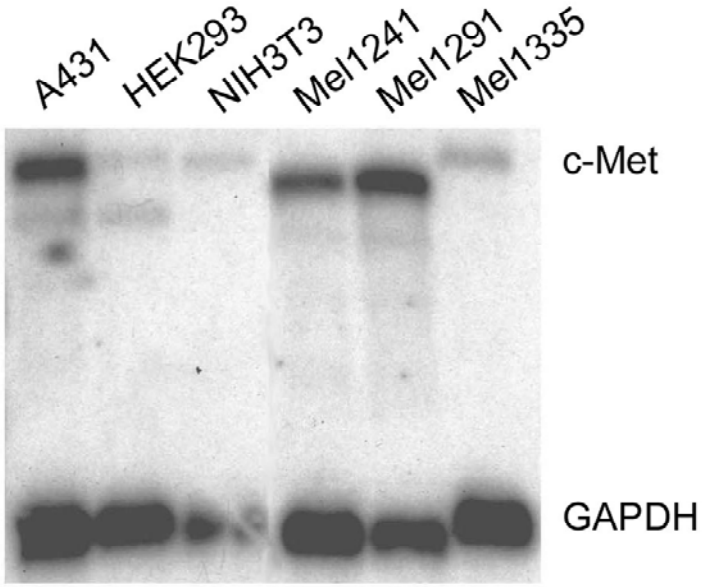
Figure 2



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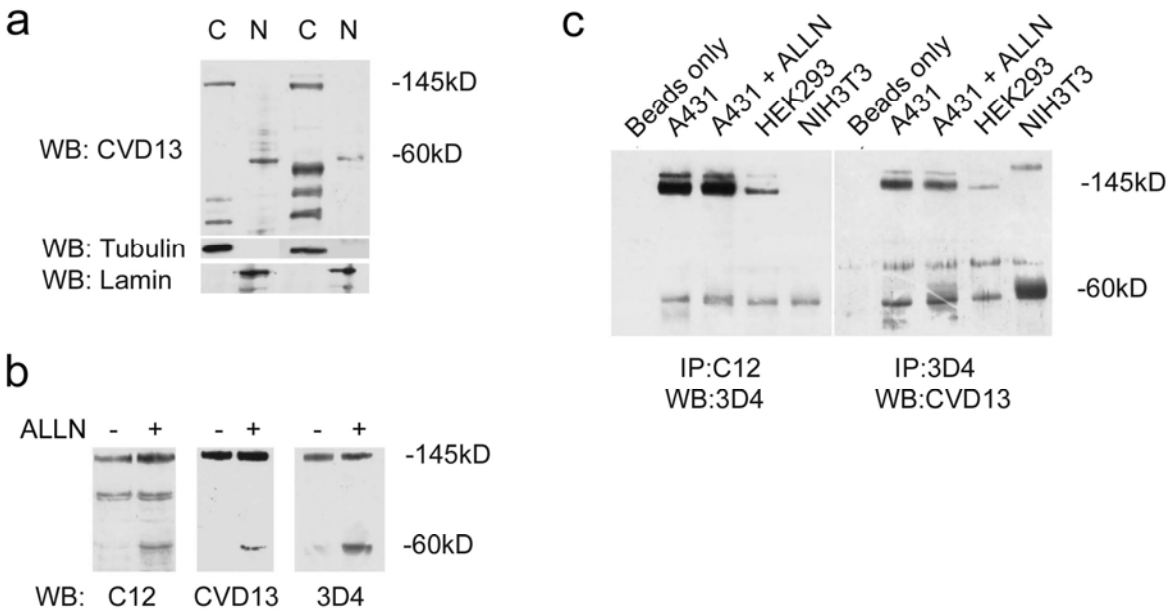

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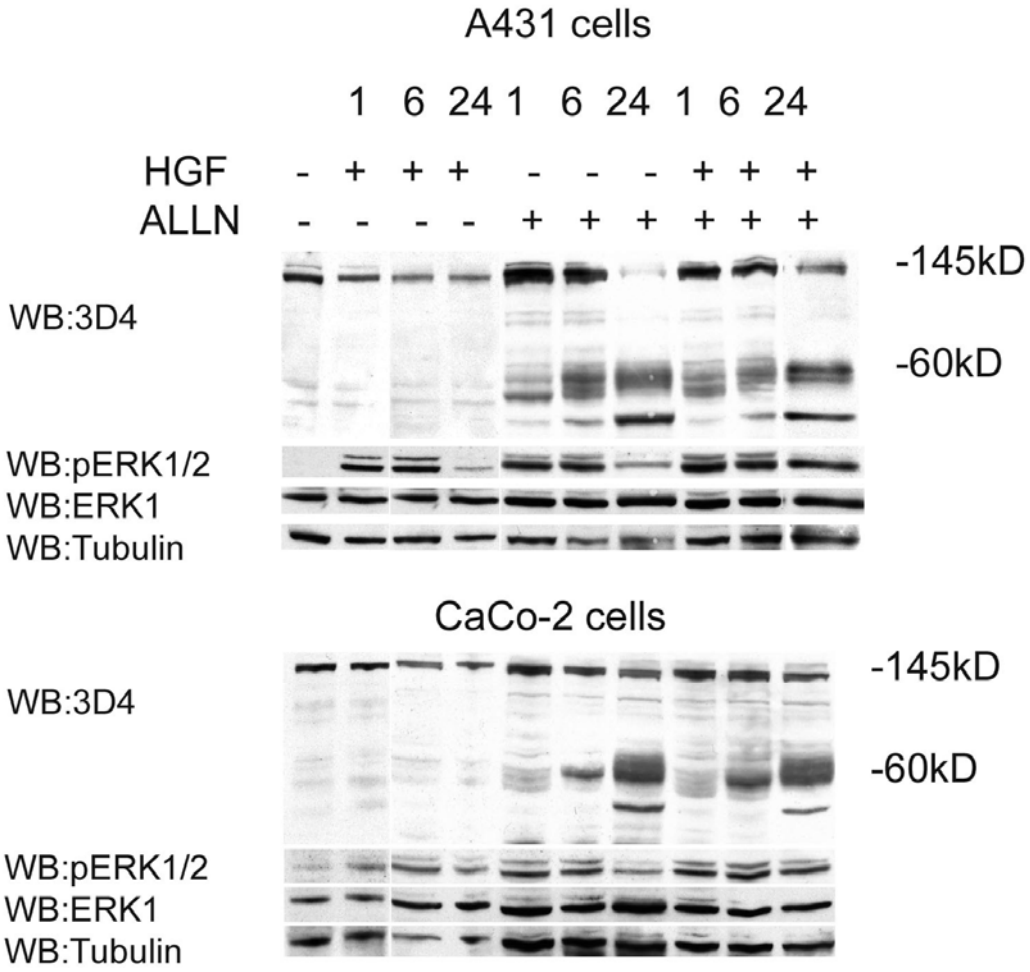
A431 cells
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HGF - + + + - - - + + +
ALLN - - - - + + + + + +
-145kD
-60kD
WB:3D4
WB:pERK1/2
WB:ERK1
WB:Tubulin
CaCo-2 cells
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-60kD
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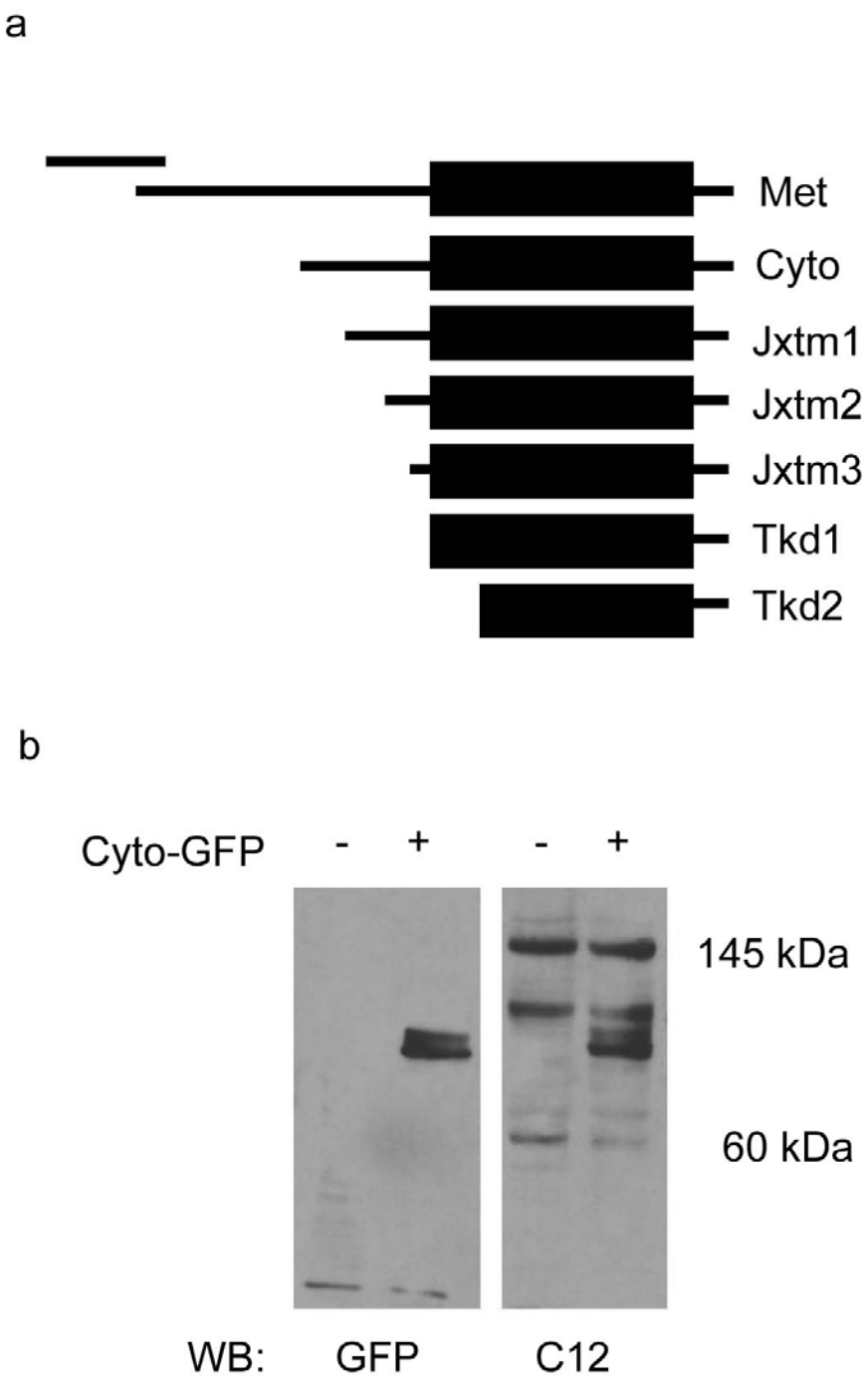
WB:ERK1
WB:Tubulin

Figure 5


a
Met
Cyto
Jxtm1
Jxtm2
Jxtm3
Tkd1
Tkd2
b
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-
+
-
+
145 kDa
60 kDa
WB:
GFP
C12

Figure 5c

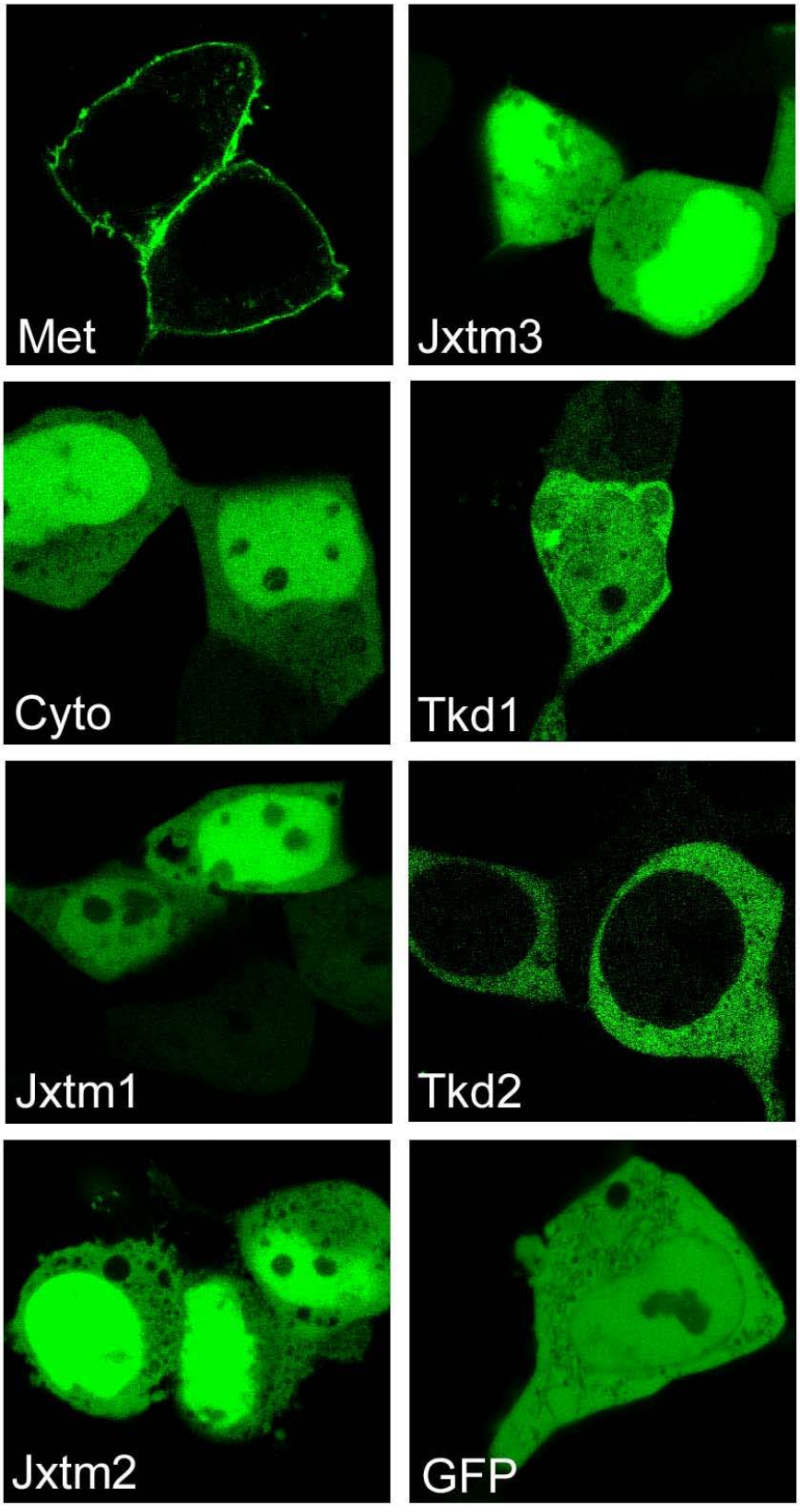

Figure 6a

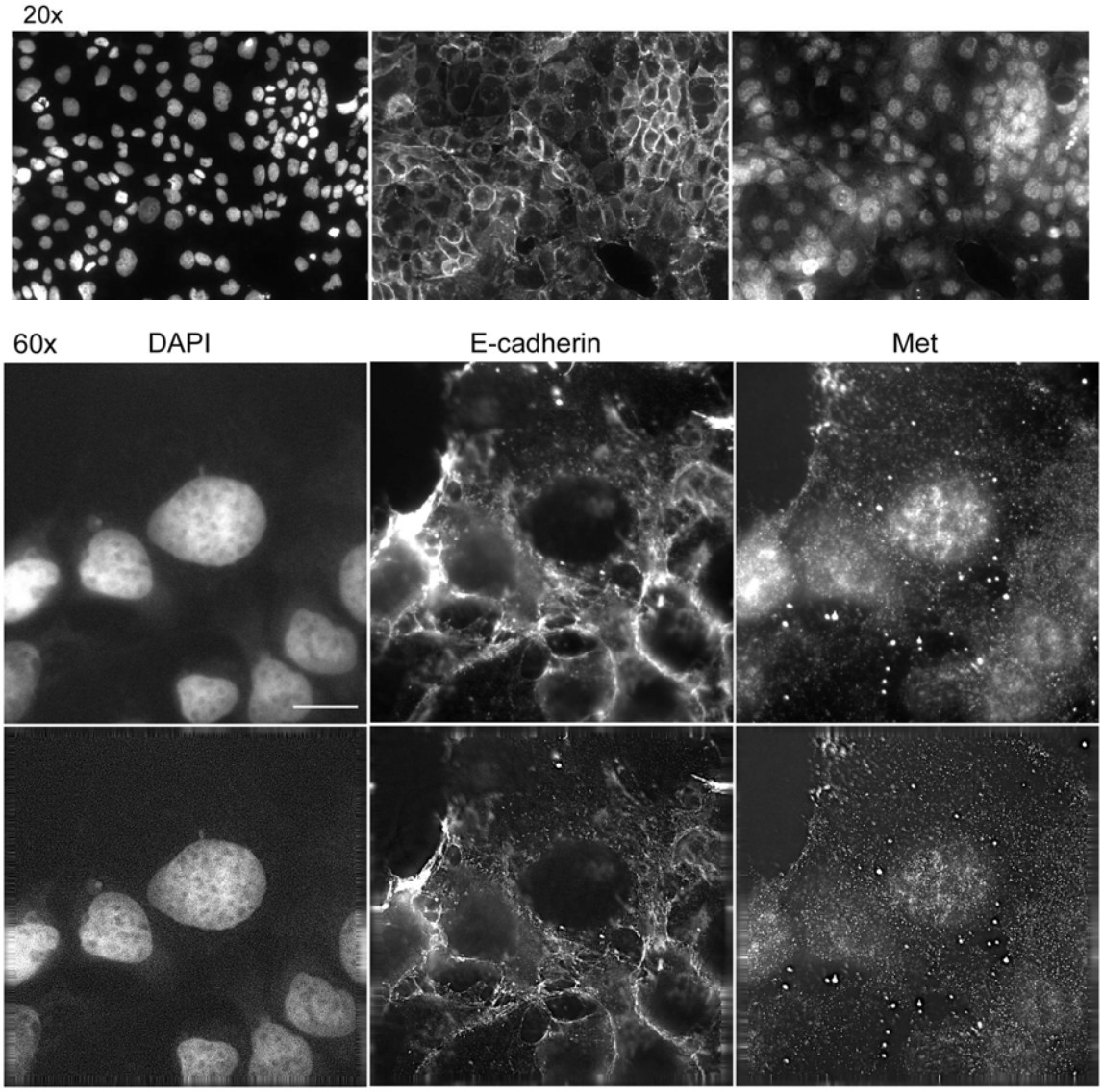

Figure 6b

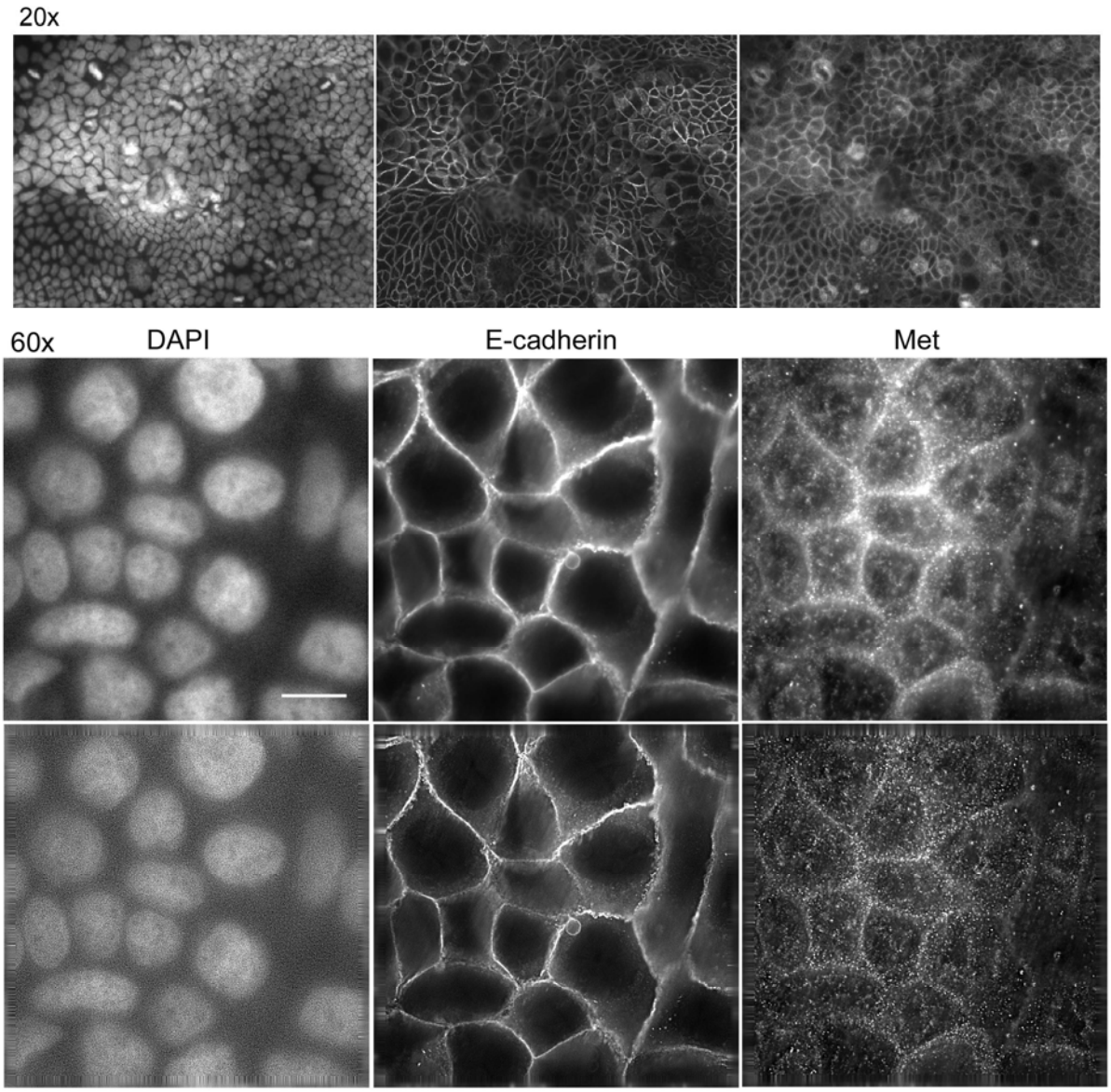

Figure 7